

Atorvastatin, Administered at the Onset of Reperfusion, and Independent of Lipid Lowering, Protects the Myocardium by Up-Regulating a Pro-Survival Pathway

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OBJECTIVES	The purpose of this study was to determine whether atorvastatin, a 3-hydroxy-3-methylglutaryl (HMG)-co-enzyme A (CoA) reductase inhibitor, limits myocardial necrosis when administered as an adjunct to reperfusion.
BACKGROUND	Statins inhibit HMG-CoA reductase to reduce the synthesis of cholesterol. However, it is proposed that statins have cardiovascular effects beyond their ability to lower cholesterol, possibly via recruitment of phosphatidyl inositol 3-kinase (PI3K) and the serine/threonine kinase, Akt. This signaling pathway has recently been linked to growth factor-mediated reperfusion salvage.
METHODS	Isolated perfused mouse hearts were subjected to 35 min of global ischemia and reperfused for 30 min in the presence of incremental concentrations of atorvastatin. Infarct size was determined by triphenyltetrazolium chloride staining, and the activity of the PI3K signaling cascade was determined by Western blot analysis.
RESULTS	We found that there was a profound dose-dependent reduction of infarct size with atorvastatin in the range of 25 to 100 $\mu\text{mol/l}$ (optimal protection was seen at 50 $\mu\text{mol/l}$ with infarct size of $16 \pm 2\%$ vs. control, $33 \pm 2\%$, $p < 0.01$). Moreover, this protection was sensitive to inhibition with the PI3 kinase inhibitor, wortmannin, and was absent in endothelial nitric oxide synthase (eNOS) knockout mice. Western blot analysis revealed that atorvastatin resulted in rapid activation of the PI3K/Akt signaling cascade (within 5 min) and that both Akt and eNOS phosphorylation were significantly increased by 4.1-fold and 2.9-fold, respectively ($p < 0.01$). Moreover, phosphorylation of the PI3K substrates was abrogated by the administration of wortmannin.
CONCLUSIONS	Atorvastatin attenuates lethal reperfusion-induced injury in a manner that is reliant on PI3K and Akt activity and the presence and activity of eNOS. (J Am Coll Cardiol 2003;41: 508–15) © 2003 by the American College of Cardiology Foundation

Reperfusion is a prerequisite to myocardial salvage after coronary occlusion. Although reperfusion is undeniably beneficial, it is perhaps not without risk. While there is some controversy regarding reperfusion injury as an event independent of the antecedent ischemia (1,2), we and others propose that this form of injury may be amenable to pharmacologic manipulation (3,4). Recently, we have identified a potential role for the phosphatidyl inositol 3-kinase (PI3K) and the downstream target, the serine/threonine kinase, Akt pathway in ameliorating reperfusion injury (5,6). Indeed, using the growth factors, insulin and insulin-like growth factor-1, it has been shown that recruitment of the PI3K/Akt signaling cascade upon reperfusion may be beneficial in limiting myocardial/myocyte cell death (5–8). Moreover, over-expression of Akt in rats attenuates cell death and myocardial dysfunction after injurious ischemia (9).

3-Hydroxy-3-methylglutaryl (HMG)-co-enzyme A

(CoA) reductase inhibitors have been increasingly prescribed for patients with cardiovascular disease after robust outcome results from both primary (10) and secondary prevention studies (11). It is, however, becoming clear that statins appear to have pleiotropic effects beyond their ability to lower cholesterol (12). Indeed, statins have been shown to be beneficial in such diverse conditions as Alzheimer's disease (13) and osteoporosis (14).

The signaling sequelae of HMG-CoA reductase remain unclear. In attenuating HMG-CoA reductase activity, statins reduce cellular content of the products of the mevalonate metabolic pathway that leads to the synthesis of cholesterol. Many of these products, the isoprenoids, are thought to be essential for the growth and proliferation of eukaryotic cells. One important isoprenoid is farnesol, which is capable of binding covalently to a number of guanosine triphosphate-binding proteins such as p21ras; a potentially important component of growth factor-stimulated signaling (15). Another is geranylgeranyl pyrophosphate, which through geranylgeranylation of the guanosine triphosphatase Rho appears to down-regulate endothelial nitric oxide synthase (eNOS) messenger ribonucleic acid (mRNA) (16). This effect may help to explain, in part, why statins lead to increased eNOS mRNA in

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Abbreviations and Acronyms

AU	= arbitrary units
DMSO	= dimethyl sulfoxide
eNOS	= endothelial nitric oxide synthase
HMG-CoA	= 3-hydroxy-3-methylglutaryl-co-enzyme A
KO	= knockout
PI3K	= phosphatidylinositol 3-kinase
TTC	= triphenyl tetrazolium chloride

thrombocytes (17) and are able to reverse the down-regulation of eNOS by low-density lipoprotein cholesterol (18). Up-regulation of eNOS activity is of potential interest in reperfusion injury; recent work with nitric oxide (NO) donors suggests that NO attenuates myocardial dysfunction after an injurious ischemia/reperfusion insult (19,20).

Recently, HMG-CoA reductase inhibitors have been shown to recruit the PI3K and Akt signaling cascade in endothelial cell lines, although not in neonatal rat cardiac myocytes (21). However, simvastatin has been shown to possess infarct sparing properties and is capable of attenuating reperfusion myocardial dysfunction in adult rats (22,23). Evidence to support a potential role of statins in recruiting the cell-survival PI3K/Akt pathway in the cardiovascular system is twofold. First, atorvastatin administration to aortic endothelium results in a decrease in caveolin abundance and, thus, reduced inhibitory binding of eNOS. This is correlated with recruitment of eNOS into an eNOS/heat stress protein 90/Akt complex, leading to the phosphorylation of eNOS at serine 1177 (24). Second, statins have been shown to have both a proliferative and anti-proliferative effect on the vasculature. At low concentrations, statins have angiogenic properties, whereas at higher concentrations they are anti-proliferative (25); the angiogenic process has been linked to the recruitment of the PI3K/Akt pathway and phosphorylation of eNOS (21).

Thus, we hypothesized that exposure of the normo-cholesterolemic adult heart to the HMG-CoA reductase inhibitor, atorvastatin, after a period of injurious ischemia, would recruit the PI3K pathway and lead to cardioprotection. Because the optimal concentration of atorvastatin that would prove to be protective was unknown, the initial aim was to construct a dose-response curve using concentrations of atorvastatin that are clinically representative of those used to reduce cholesterol in hypercholesterolemic patients. Secondary aims were to determine whether there was a role for the PI3K/Akt signaling cascade, whether recruitment of eNOS occurs, and whether the activity of this pathway is essential to HMG-CoA reductase inhibitor-induced protection.

METHODS

All work was conducted in accordance with the Guidelines on the Operation of the Animals (Scientific Procedures) Act

1986, published by The Stationery Office (London, UK). Wortmannin was purchased from Sigma (Poole, UK). Dimethyl sulfoxide (DMSO; BDH Laboratory Supplies, Poole, UK) was used as the solvent for wortmannin and used at a final concentration in the perfusion buffer of 0.001%. Atorvastatin was kindly supplied by Pfizer and was dissolved in methanol (BDH Laboratory Supplies, Poole, UK) at 5 mg/100 μ l. Final concentration of methanol in the perfusion buffer did not exceed 0.001%.

Mice were obtained from the breeding colony kept at University College, London, bred under license from Jackson Laboratories. Both eNOS wild-type (parental wild-type, based on C57BL/6J background) and eNOS knockout (KO) mice were used in the present study; a total of 75 mice were used. Three mice were excluded before starting the experimental protocol. No hearts were excluded after the onset of the ischemia/reperfusion protocol.

Langendorff perfusion. Male and female eNOS KO mice (3 to 4 months of age, 20 to 30 g weight) were randomly attributed to either control or treatment groups. Heart isolation and Langendorff perfusion (with modified Krebs-Henseleit buffer consisting of NaCl 118 mmol/l, NaCO₃ 24 mmol/l, glucose 10 mmol/l, KCl 4 mmol/l, NaH₂PO₄ 1.0 mmol/l, Na₂ ethylenediaminetetraacetic acid 0.5 mmol/l, MgCl₂ 1.2 mmol/l, CaCl₂ 2.5 mmol/l) were performed as previously described (26). In brief, mice were anesthetized with an intra-peritoneal injection of 60 mg/kg pentobarbitone. Hearts were then harvested via a para-medial thoracotomy and rapidly transferred to a dissection dish filled with ice cold Krebs-Henseleit buffer. The aorta was cannulated with a 21-gauge murine cannula and transferred to the Langendorff rig. Hearts were paced throughout the stabilization period and during the final 20 min of reperfusion (600 beats/min).

Western blotting. Western blot analysis was performed as previously described (26). Lanes were loaded at 60 μ g/lane. Loading was confirmed both by Ponceau staining of the membranes after transfer from the polyacrylamide gel and by quantification of the relevant non-phosphorylated protein (Akt or eNOS) by Western blot analysis. The membranes were probed with rabbit polyclonal antibodies for Akt, phospho- (serine 473) Akt, eNOS, and phospho- (serine 1177) eNOS antibodies purchased from Cell Signalling (Hitchin, UK) and used in accordance with the manufacturer's instructions. Quantification was achieved using the enhanced chemiluminescence technique, with exposure to photographic film. The developed films were scanned using a flatbed document scanner, and the relative densitometry was assessed using the National Institutes of Health (NIH) Shareware program, NIH Image 1.62.

Experimental protocols. Langendorff perfusions were split into two phases: phase A for infarct size analysis and phase B for Western blot analysis. All hearts were subjected to 35 min of global, normothermic ischemia. Hearts were then either reperfused for 30 min and stained with triphenyl

tetrazolium before planimetry in phase A, or they were reperfused for 5, 10, or 15 min and snap-frozen in phase B. **PHASE A.** The eNOS wild-type and eNOS KO hearts were randomized to receive one of the following treatments from the onset of reperfusion: 1) vehicle ($n = 6$ each for Krebs-Henseleit buffer, 0.001% methanol, or 0.001% DMSO); 2) atorvastatin ($n = 6$ each for 5, 25, 50, and 100 $\mu\text{mol/l}$); and 3) wortmannin 100 nmol/l in the presence or absence of 25 $\mu\text{mol/l}$ atorvastatin.

PHASE B. These experiments were divided into two groups as follows: 1) The eNOS wild-type hearts were randomized into 5-, 10-, or 15-min reperfusion groups in the presence or absence of 25 $\mu\text{mol/l}$ atorvastatin; 2) The eNOS wild-type hearts were randomized to receive either vehicle or 25 $\mu\text{mol/l}$ atorvastatin in the presence or absence of wortmannin, 100 nmol/l, whereas eNOS KO hearts were randomized to receive either vehicle or 25 $\mu\text{mol/l}$ atorvastatin.

Infarct size analysis. Infarct size was determined by the triphenyl tetrazolium chloride (TTC) staining technique as previously described (26). In brief, the hearts at the end of the experimental protocol were infused with TTC in phosphate-buffered solution (pH 7.4). This turns viable tissue a dark red color, whereas non-viable, necrotic myocardium appears pale. The hearts were incubated for 10 min in TTC before destaining in 10% formaldehyde solution to increase contrast between necrotic and viable myocardium. Infarct size is expressed as an infarct-to-risk-zone ratio (when the risk zone in the global ischemic heart is the whole ventricular volume). Data are presented as a percentage \pm SEM.

Statistical analysis. All values are expressed as mean \pm SEM. Differences in continuously distributed variables between predetermined experimental groups were analyzed using one-way analysis of variance followed by Fisher's protected test of least significant difference. A p value of 0.05 was considered to be at the limit of statistical significance.

RESULTS

Does atorvastatin limit infarct size when administered as an adjunct to reperfusion? To determine whether atorvastatin is capable of attenuating myocardial infarction (MI) in this isolated mouse model of ischemia and reperfusion, we compared wild-type hearts, in the presence or absence of the solute vehicle, methanol (0.001%) added at reperfusion, with incremental concentrations of atorvastatin administered upon reperfusion. Over a concentration range of 25 to 100 $\mu\text{mol/l}$, atorvastatin significantly attenuated infarction ($p < 0.01$), with maximal protection observed with the 50 $\mu\text{mol/l}$ dose. Infarct-to-risk-zone ratios were $26 \pm 3\%$, $19 \pm 2\%$, $16 \pm 2\%$, and $21 \pm 3\%$ for 5, 25, 50, and 100 $\mu\text{mol/l}$, versus Krebs-Henseleit and methanol vehicle control $32 \pm 2\%$ and $33 \pm 2\%$, respectively (Fig. 1).

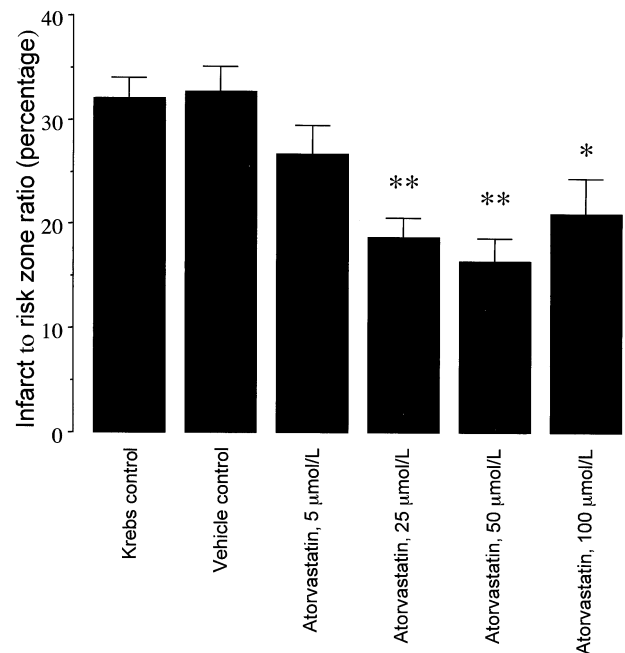


Figure 1. Atorvastatin dose-response curve. Incremental concentrations of atorvastatin resulted in a dose-dependent reduction of infarct size, peaking with 50 $\mu\text{mol/l}$ (** $p < 0.001$ vs. control). Protection with 100 $\mu\text{mol/l}$ was, however, less than that seen at 50 $\mu\text{mol/l}$ (* $p < 0.05$ vs. control).

Does atorvastatin administration at reperfusion result in increased activity of PI3K and Akt? To determine whether atorvastatin-mediated reperfusion salvage is mediated by the recruitment of the PI3K/Akt signaling cascade, we measured the phosphorylation of the substrates of each of these protein kinases, Akt and eNOS, respectively. In control hearts, modest phosphorylation of Akt and eNOS was observed 10 min after the onset of reperfusion (Figs. 2 and 3), although over the time period examined (15 min of reperfusion) there was no statistically significant increase in the activity of either PI3K or Akt. In contrast, treatment of the hearts at reperfusion with 25 $\mu\text{mol/l}$ atorvastatin resulted in a significant increase of phosphorylation at all time points measured, suggesting that atorvastatin results in a rapid and more robust activation of the PI3K signaling cascade. Densitometry data indicate that the total Akt and eNOS phosphorylation after reperfusion in the atorvastatin group were 4.1-fold and 2.9-fold greater, respectively ($p < 0.001$), than over the same 15-min period in control hearts. Performing a simple regression plot between the quantification of phospho-Akt and phospho-eNOS resulted in a robust correlation ($r^2 = 0.7$, $p < 0.001$ with a gradient of 0.8) indicating a direct relationship between the phosphorylation of Akt and the phosphorylation of eNOS.

Is increased PI3K activity essential for reperfusion salvage, or an epiphenomenon? To determine whether the increase in PI3K activity is functionally related to atorvastatin reperfusion salvage or merely an epiphenomenon, we used the PI3K inhibitor, wortmannin (100 nmol/l) upon reperfusion in the presence or absence of atorvastatin (25 $\mu\text{mol/l}$). Wortmannin alone had no impact on infarct

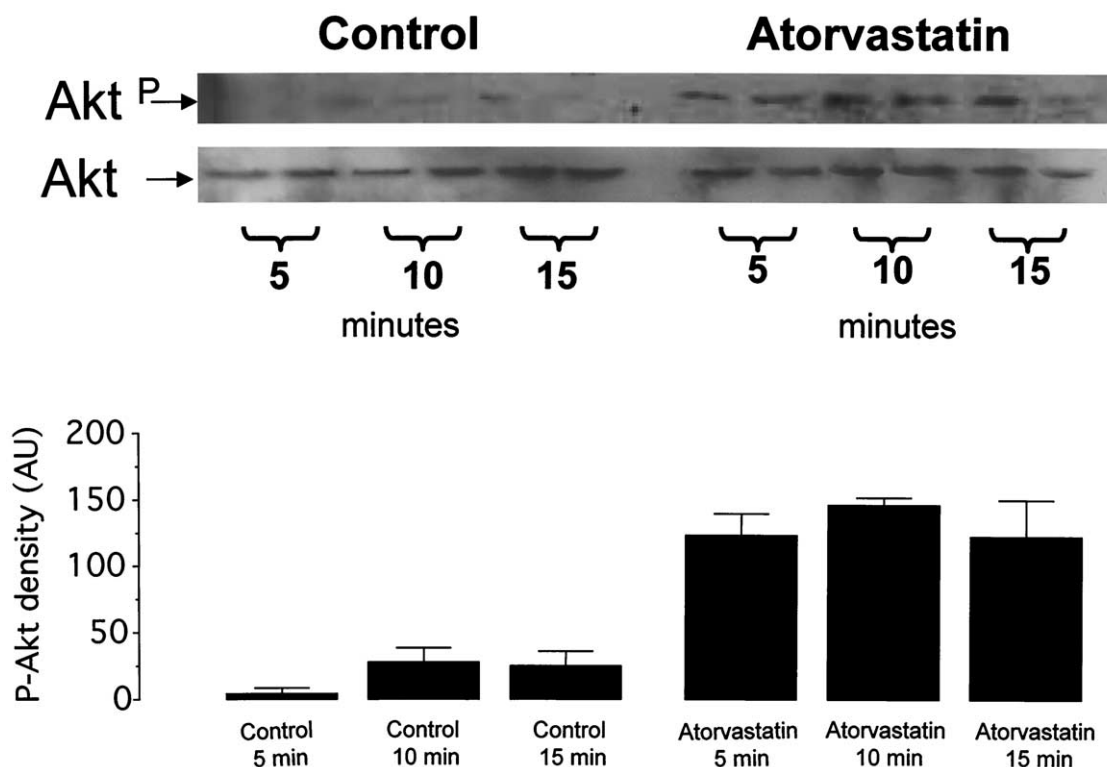


Figure 2. The Akt phosphorylation time-course. Administration of 25 $\mu\text{mol/l}$ atorvastatin to wild-type hearts at the start of reperfusion after a period of ischemia resulted in a robust and rapid increase in Akt phosphorylation (4.1-fold greater than that seen in control hearts, $p < 0.001$).

size ($32 \pm 2\%$ vs. control, $34 \pm 2\%$). However, wortmannin completely abrogated the protection observed with atorvastatin ($33 \pm 2\%$ vs. atorvastatin alone, $19 \pm 2\%$ and control $34 \pm 2\%$, $p < 0.05$, Fig. 4).

Is eNOS required for atorvastatin-mediated protection? To determine whether eNOS is essential in the mediation of atorvastatin reperfusion salvage, we administered the cardioprotective dose of atorvastatin (25 $\mu\text{mol/l}$) to eNOS KO hearts. In contrast to the eNOS wild-type hearts previously studied, we found no protection ($32 \pm 1\%$ vs. control, $31 \pm 1\%$, Fig. 5).

Is PI3K activity modified in eNOS KO hearts and inhibited by wortmannin? To determine whether the loss of atorvastatin-mediated protection that was observed in eNOS KO hearts and eNOS wild-type hearts treated with wortmannin was mediated by inhibition of PI3K activity, we assayed Akt phosphorylation in KO and wortmannin-treated hearts. Wortmannin administration significantly attenuated atorvastatin-triggered Akt phosphorylation to below the level seen in control hearts (143 ± 6 AU vs. atorvastatin alone, 772 ± 31 AU and control, 422 ± 52 AU, $p < 0.001$ and $p < 0.01$, respectively, Fig. 6).

Although no protection was observed as a result of atorvastatin administration in eNOS KO hearts, a similar level of Akt phosphorylation was nonetheless observed in KO hearts as it was in wild-type hearts (866 ± 32 AU vs. wild-type atorvastatin-treated hearts, 772 ± 31 AU, $p > 0.05$). Thus PI3K activity is indeed increased by

atorvastatin-treated hearts despite the absence of cardioprotection.

DISCUSSION

This study identifies the HMG-CoA reductase inhibitor, atorvastatin, as a drug capable of attenuating MI when administered as an adjunct to reperfusion, in a setting that is independent of cholesterol lowering. The data demonstrate, for the first time, that atorvastatin administered as an adjunct to reperfusion results in significant, dose-dependent cardioprotection, with an optimal concentration range between 25 and 100 $\mu\text{mol/l}$ and maximal protection at 50 $\mu\text{mol/l}$. This study also identifies a key signaling pathway by which this protection at reperfusion is wrought: the PI3K signaling cascade—the first time that statins have been shown to regulate this kinase pathway in myocardium. Previous work has shown that statins may have quite opposing effects on tissue Akt activation and cellular survival, with up-regulation demonstrated in endothelial cells (21), no effect on neonatal myocytes (21), and a down-regulation in smooth muscle cells (27,28). Interestingly, our preliminary data in unstressed, Langendorff perfused, adult mouse myocardium indicated that brief (10-min) administration of 25 $\mu\text{mol/l}$ atorvastatin did not enhance PI3K activity, as determined by the quantification of Akt phosphorylation by Western blotting (data not shown). However, we show here that 25 $\mu\text{mol/l}$ atorvastatin does signif-

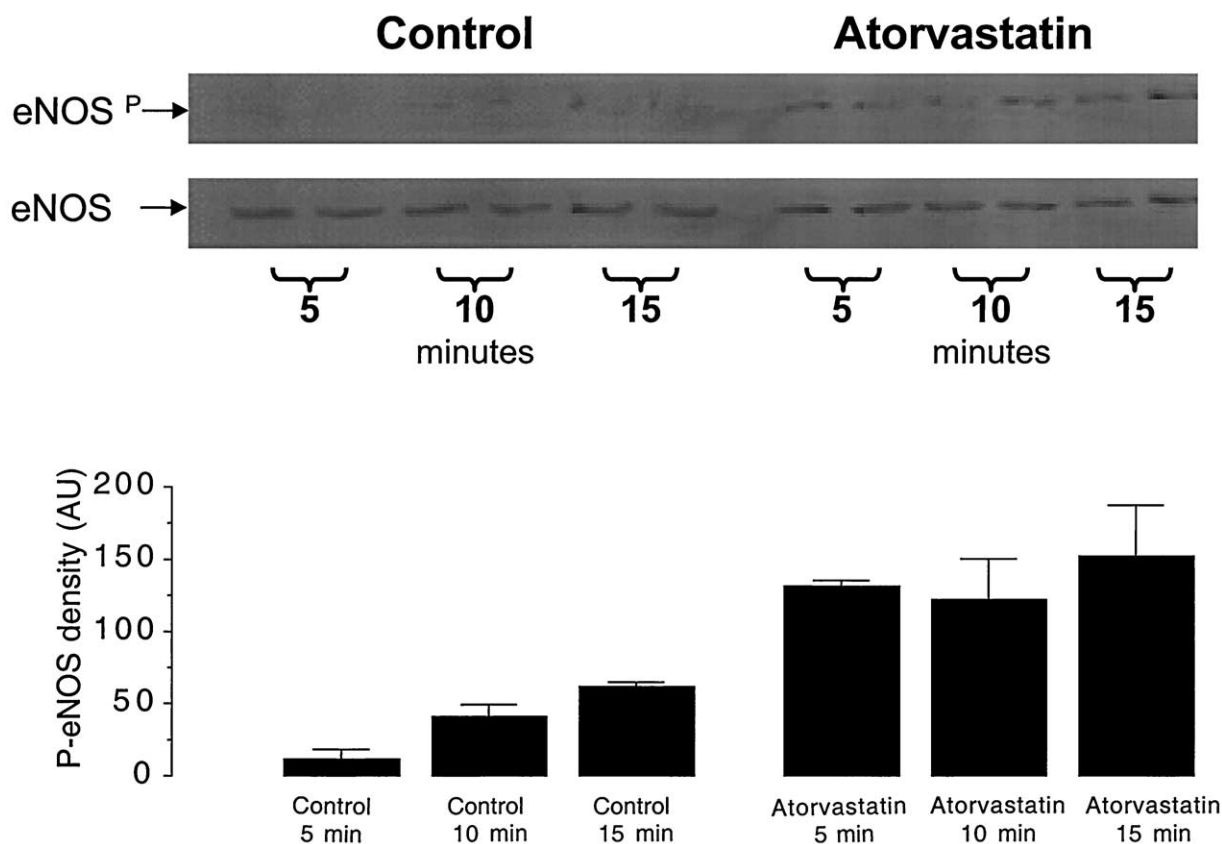


Figure 3. The eNOS phosphorylation time-course. Administration of 25 $\mu\text{mol/l}$ atorvastatin at the start of reperfusion after a period of ischemia resulted in a similar pattern of phosphorylation of eNOS to that seen with Akt, with a significant 2.8-fold increase over control ($p < 0.001$).

icantly increase phosphorylation of both Akt and eNOS in the post-ischemic myocardium (although the specific cell types have not been delineated). These data provide evi-

dence that the PI3K may be playing an important role during the early reperfusion period, as has been suggested by recent work by ourselves and others with insulin both in

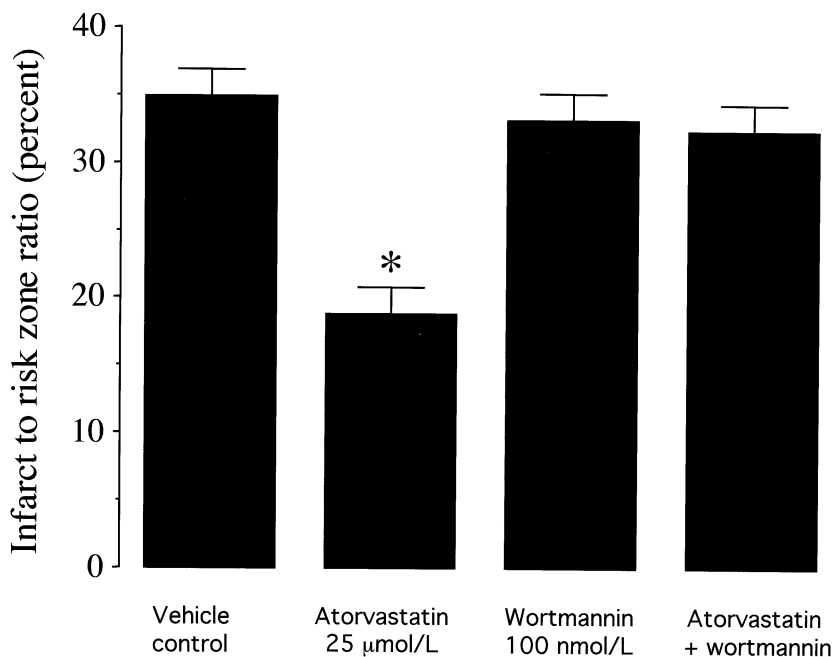


Figure 4. The PI3K inhibitor, wortmannin, inhibits the reperfusion salvage seen with 25 $\mu\text{mol/l}$ atorvastatin. Wortmannin administered alone had no impact on infarction (* $p < 0.05$, atorvastatin group vs. control and wortmannin plus atorvastatin).

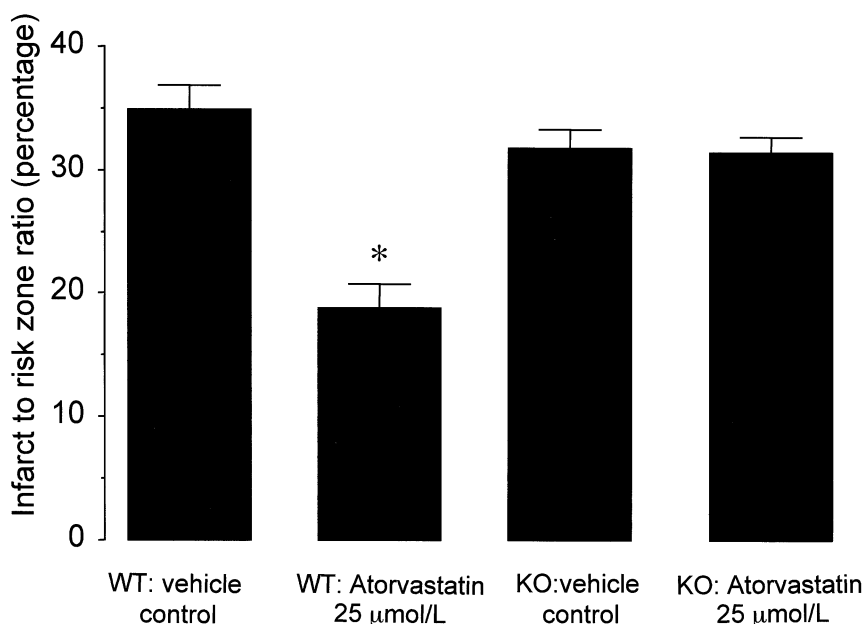


Figure 5. Reperfusion salvage seen with 25 µmol/l atorvastatin is absent in eNOS KO mice. Although atorvastatin results in significant protection in the wild-type hearts (WT), this protection was completely absent in the eNOS KO hearts (* $p < 0.001$ atorvastatin vs. WT control).

vitro and in vivo (6,29). The PI3K hypothesis of reperfusion salvage is further supported by the data obtained after the administration of the PI3K inhibitor wortmannin. Not only does wortmannin lead to a significant attenuation of Akt phosphorylation associated with atorvastatin administration, wortmannin also abrogates atorvastatin-mediated protection. Thus, it appears that PI3K activity is essential in attenuating reperfusion injury.

A significant number of downstream targets for the PI3K signaling cascade have been identified (30), although those that are essential for early reperfusion salvage are not clear. One candidate that we have recently identified is the ribosomal p70S6 kinase (6). In this study, using insulin to trigger reperfusion salvage via insulin tyrosine kinase linked receptor, we were able to maintain the pro-apoptotic Bcl-2 family member, Bad, in a phosphorylated and, therefore, inactivated state (6,31). In the present study, we have investigated the role of an alternative target of Akt, namely eNOS (32). Concurrent with the rapid increase in Akt phosphorylation observed in hearts treated with atorvastatin administered at reperfusion, we see a significant and parallel increase of the phosphorylation of eNOS. Comparing the phosphorylation densitometry of both Akt and eNOS blotted from the same membrane, we find a significant and high correlation between the two events, implying that phosphorylation of eNOS is closely related to phosphorylation and activity of Akt. Significantly, the protection wrought by the administration of atorvastatin is completely absent in the eNOS KO heart. This suggests that eNOS is pivotal to the PI3K signaling cascade; Western blot analysis demonstrates that eNOS KO hearts are not deficient in the activity of PI3K, as measured by Akt phosphorylation. Therefore, it would seem that in this model of ischemia and

reperfusion, PI3K activation alone is not sufficient to provide protection against infarction in the reperfused heart.

The concentrations of atorvastatin used in the present investigation are representative of top-end oral administration of the drug used in the management of hypercholesterolemic patients. Lower concentrations of atorvastatin have been found to be beneficial in promoting angiogenesis; it is interesting to note, however, that the concentrations used in the present study are anti-angiogenic according to recent work by Weis et al. (25). The duration of administration is the determining factor in producing this effect; in the present model we are investigating the immediate direct myocardial protective properties of statins, whereas the angiogenic effects are found with long-term administration, suggesting a dichotomy of beneficial concentration depending on the period of the treatment regime. That a "high dose" of statin is appropriate in the short-term setting of reperfusion injury management is supported by the recent work of Di Napoli et al. (22) with 25 µmol/l simvastatin in a working heart model of ischemia/reperfusion. In that study, simvastatin was administered with the onset of 15 min of global ischemia and at 180 min of reperfusion; high-dose simvastatin was found to be cardioprotective and, moreover, dependent on NO synthase activity.

Isolated, crystalloid perfused hearts were used in the present study to remove the potentially confounding effects of serum cholesterol, of actions on thrombocytes, and of the neutrophil/endothelial interaction previously hypothesized to be the mechanism of statin-mediated protection (17,33). In this study, we demonstrate that atorvastatin is capable of conferring protection on the reperfused myocardium independently of these factors, suggesting a direct effect on the myocardium of increasing the resistance to ischemia/

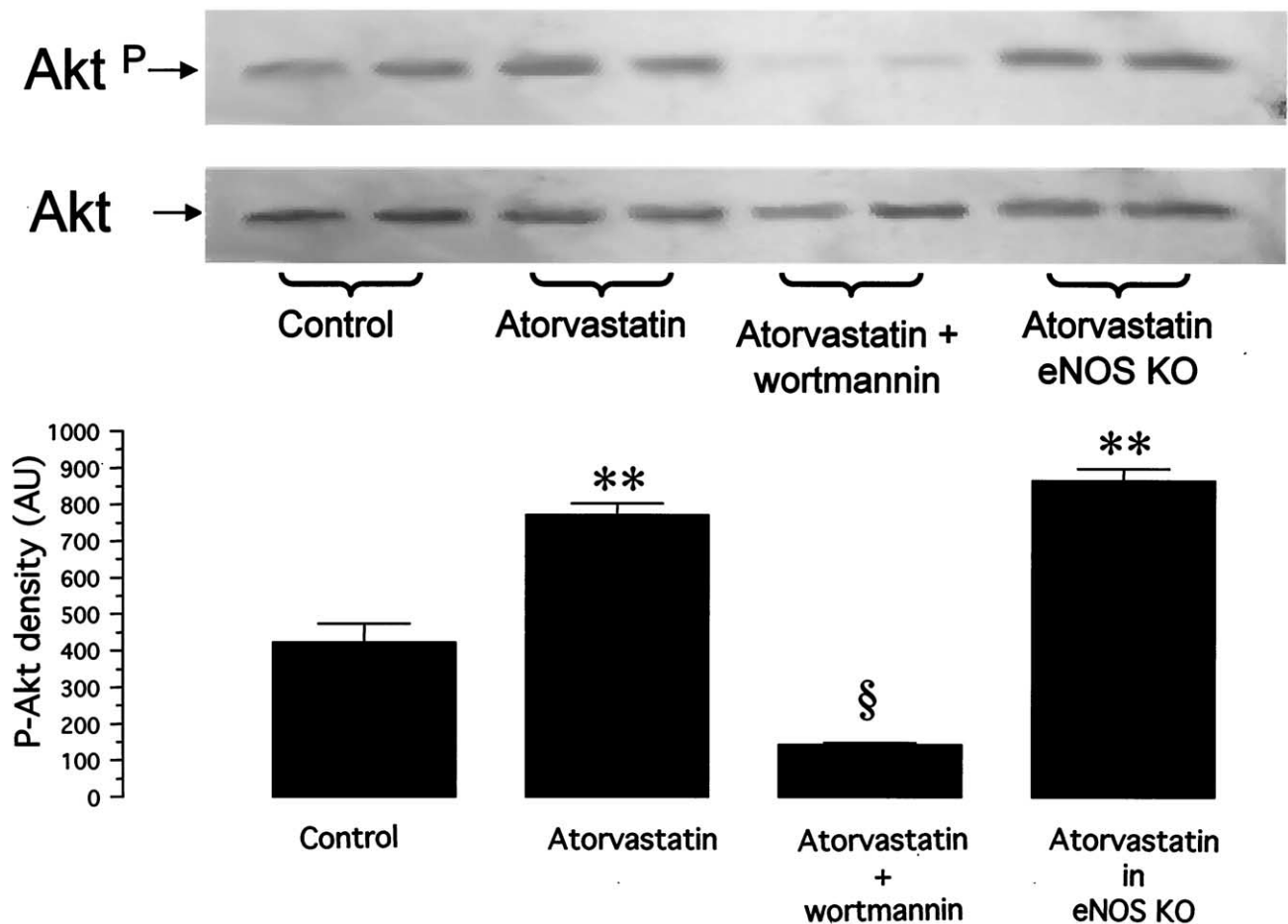


Figure 6. Akt phosphorylation with atorvastatin after 10 min of reperfusion: the effect of wortmannin and the absence of eNOS. Administration of atorvastatin to wild-type and KO hearts resulted in a robust increase in phosphorylation of Akt, although no cardioprotection is seen in eNOS KO hearts (** $p < 0.001$ vs. control hearts). The administration of the PI3K inhibitor, wortmannin, significantly reduced Akt phosphorylation compared with both control and atorvastatin-treated hearts (§ $p < 0.01$ vs. both control and atorvastatin groups).

reperfusion injury, mediated by PI3K, Akt, and eNOS. However, the isolated perfused model of ischemia and reperfusion has limitations specifically related to the limited time during which an isolated heart may be reperused. Although this limitation should be recognized in the context of evolving MI and remodeling, we believe that the data presented here provide an insight into the mechanisms by which statins appear to confer additional protection on the ischemic heart beyond the drugs' ability to reduce serum cholesterol (34,35).

In summary, we present evidence that the HMG-CoA reductase inhibitor, atorvastatin, is capable of attenuating ischemia/reperfusion injury when administered at the onset of reperfusion. Moreover, we show that the mechanism of action by which this protection is achieved is dependent on the recruitment and activation of a signaling cascade involving PI3K, Akt, and eNOS. Moreover, this protection appears to be mediated directly via the myocardium, independently of circulating thrombocytes, neutrophils, and other inflammatory moieties, and independently of a cholesterol-lowering effect.

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